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A Pathogenic Role of Th2 Cells and Their Cytokine Products on the Pulmonary Metastasis of Murine B16 Melanoma

Makiko Kobayashi, Hiroyuki Kobayashi, Richard B. Pollard, and Fujio Suzuki

The role of Th2 cells and the cytokines produced by these cells on experimental pulmonary metastasis of B16 melanoma was investigated in a murine model implanted with high metastatic (B16F10) or low metastatic (B16F1) melanoma cells. An average of 250 colonies of metastasis in the lungs was counted in mice (BF10 mice) at 14 days after the inoculation of $2 \times 10^5$ B16F10 cells/mouse, while <20 colonies were detected in mice (BF1 mice) inoculated with the same number of B16F1 cells. CD4$^+CD11b^-TCR-\alpha\beta^+$ T cells (BF10-Th2 cells) were produced in the spleens of BF10 mice, while these cells were not detected in BF1 mice. The BF10-Th2 cells produced IL-4 and IL-10 into culture fluids when stimulated in vitro with anti-CD3 mAb. However, IL-2 and IFN-\(\gamma\) were not produced. The level of a pulmonary metastasis in BF1 mice increased to the level observed in BF10 mice, when BF10-Th2 cells were adoptively transferred to BF1 mice. Also, an increase in the number of pulmonary melanoma was demonstrated in BF1 mice treated with 10 \(\mu\)g/kg murine rIL-4. The level of pulmonary metastasis in BF10 mice or in BF1 mice inoculated with BF10-Th2 cells decreased to the level observed in BF1 mice when mice were treated with an anti-IL-4 mAb at a dose of 250 \(\mu\)g/kg on days 1, 3, and 5 after tumor inoculation. These results suggest that the severity of pulmonary metastasis in mice receiving B16 melanoma cells is strongly influenced by the IL-4 released from tumor-associated Th2 cells.


The control of suppressed cell-mediated immunity, which includes decreased functionality of antitumor effector cells (activated macrophages, NK cells, lymphokine-activated killer cells, and tumor-specific cytotoxic T cells), on accelerating tumor metastasis has been described previously (1–10). Soluble tumor Ags (4), formation of circulating immune complexes (5), and immunosuppressive cytokines, such as TGF-\(\beta\) and IL-10 production by tumor cells (6–9), have been shown to influence the tumor-associated suppression of cell-mediated immunity. Immunosuppressive soluble factors, such as \(\alpha\)-acid glycoprotein and 1-methyladenosine produced by either the host’s immunocompetent cells or tumor cells, have also been shown to be involved in tumor-related immunosuppression (11). Type 1 T cell responses enhance cellular immune responses associated with increased levels of type 1 cytokines (IL-2 and IFN-\(\gamma\)) and the generation of CTL (a typical effector cell of type 1 T cell responses). In most instances, antitumor immunity is produced by the activation of type 1 T cell responses (12, 13). Therefore, the suppression of type 1 T cell responses can result in the acceleration of tumor growth. Type 1 T cell responses are often suppressed by T helper type 2 cells (Th2 cells) that can be demonstrated in tumor-bearing mice (14–16). Type 2 cytokines (IL-4 and IL-10) released from type 2 T cells are inhibitors of type 1 T cell responses (14–16). Also, the effector cell activity of type 1 T cells and the production of type 1 cytokines (IFN-\(\gamma\), IL-2) by type 1 T cells have been shown to be suppressed by type 2 cytokines (14–16). This suggests the possibility that a contributor to the decreased effectiveness of immunotherapy in patients with malignancies might be associated with the activity of type 2 cytokines released from tumor-associated type 2 T cells. In the present study, the role of IL-4 and/or tumor-associated Th2 cells on tumor metastasis was investigated in mice inoculated with B16 melanoma cells of either high (B16F10 melanoma cells) or low (B16F1 melanoma cells) metastatic characteristics. Poste and Nicolson (17) have described the metastatic characteristics of B16F10 cells and B16F1 cells in the lung capillary beds of mice. In their article, B16F10 cells had 114 ± 24 pulmonary metastases when they were inoculated into C57BL/6 mice at a inoculum size of 5 × 10$^4$ cells/mouse. However, only 16 ± 7 pulmonary metastases were produced when C57BL/6 mice were inoculated with the same number of B16F1 cells. Additional points that distinguish differences between high metastatic B16F10 cells and low metastatic B16F1 cells are: generation speed (18); i.e. collagenase production (19); adherence to poly(hydroxyethylmethacrylate)-coated plates (20); membrane-bound protein kinase levels (21); antigenic properties (22).

Materials and Methods

Animals

Eight-week-old C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were used in these experiments. All experiments were approved by the Animal Care and Use Committee (ACUC) of the University of Texas Medical Branch at Galveston (ACUC approval number, 89-03-066).

Reagents

Anti-CD11b, anti-CD28, anti-TCR-\(\alpha\beta\), anti-TCR-\(\gamma\delta\), anti-IL-8, mAbs along with anti-IFN-\(\gamma\), anti-IL-2, IL-4, and anti-IL-10 mAbs for ELISA were purchased from PharMingen, San Diego, CA. Murine rIFN-\(\gamma\), rIL-2, rIL-4, and rIL-10 were obtained from Genzyme, Cambridge, MA. Anti-CD3 mAb was purchased from Boehringer-Mannheim Biochemicals, Indianapolis, IN. Anti-L3T4 and anti-Lyl-2.2 mAbs were obtained from Accurate Chemical and Scientific, Westbury, NY. Anti-mouse Ig (anti-Ig) antisemur was purchased from Cappel Laboratory, Cochranville, PA, and Low-Tox-M rabbit complement was obtained from Cedarlane Laboratories, Hornby, Ontario, Canada.

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1 Address correspondence and reprint requests to Dr. Fujio Suzuki, Department of Internal Medicine, The University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-0835.

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Preparations of splenic lymphocytes

As described previously (23), splenic mononuclear cells were prepared from the spleens of mice inoculated with B16 melanoma cells or controls. To prepare CD4+ T cells, splenic mononuclear cells (1 × 10^7 cells/ml) were passed through a CD4 T cell subset column (R&D Systems, Minneapolis, MN) (24). The cells obtained were washed with serum-free RPMI 1640 medium and used in the experiments. When CD4+ T cell fractions were treated with anti-L3T4 mAb followed by complement, 96% of viable cells were lysed. When these cell fractions were treated with anti-Lyt-2.2 mAb followed by complement, 2% of viable cells were lysed (24). These results suggested that the purity of the CD4+ cell preparation was 96% or greater. For phenotypical analysis, CD4+ T cells purifified from spleens of tumor-bearing mice were treated with various mAbs (4°C, 30 min) followed by complement (1/30 dilution, 37°C, 30 min) (24). The residual cells were then assayed for their ability to produce IL-4 (see Assay of cytokines). mAbs used for the phenotypic analysis included anti-CD41b (1/100 dilution), anti-CD11b, anti-L3T4 (1/100 dilution), and anti-TCR-γδ mAbs (1/100 dilution) (24).

Mice implanted with B16F1 or B16F10 melanoma cells

B16F1 cells (a low metastatic strain of B16 melanoma cells) and B16F10 cells (a high metastatic strain of B16 melanoma cells), provided by Tohoku University School of Medicine, Sendai, Japan, were grown in vitro with RPMI 1640 medium supplemented with 5% FBS, antibiotics, and 1% l-glutamine (complete medium) and stored in liquid nitrogen (25). For their use in animal experiments, these cells were passed two to five times with complete medium after their regrowth from frozen stocks. Cells in a log growth phase were detached from tissue culture flasks using a mixture of 0.25% trypsin and 0.03% EDTA. The cells were then washed and suspended in PBS just before implantation. The mice were injected i.v. with 0.2 ml of the cell suspension. In these experiments, the mice were inoculated with 2 × 10^6 cells/mouse of cultured B16F1 cells or B16F10 cells, and designated as BF1 mice or BF10 mice, respectively. As required, variable numbers of splenic CD4+ T cells from normal mice (naive T cells), BF1 mice, or BF10 mice were adoptively transferred i.v. to BF1 mice 3 days after tumor inoculation. Fourteen days after tumor inoculation, lung tissues were removed from these mice and fixed in 10% formaldehyde solution (25). The number of black metastatic colonies in lungs were counted under a dissecting microscope, as described previously (25). Throughout all experiments, metastatic colonies were demonstrated only in the lungs (not other organs) of BF1 mice and BF10 mice 2 wk after tumor inoculation.

Assay of cytokines

To induce IFN-γ, IL-2, IL-4, and IL-10 in vitro, 2 × 10^6 CD4+ T cells/ml from BF1 mice or BF10 mice were stimulated with anti-CD3 mAb (2.5 μg/ml) for 48 h at 37°C (26). The culture fluids were harvested and assayed for cytokines by specific ELISA according to standard protocol. In these assays, the detection limit for cytokines using ELISA was between 5 and 50 pg/ml.

Statistical analysis

The results of the pulmonary metastasis and the cytokine assays were statistically analyzed using Student’s t test. If the p value was < 0.05, the result was considered to be significant.

Results

Generation of Th2 cells in BF10 mice

The number of pulmonary lesions of B16 melanoma cells in BF1 mice and BF10 mice at various days after the tumor cell inoculation is shown in Figure 1. Metastatic colonies of B16 melanoma cells were first detected in BF10 mice 6 days after the inoculation of B16F10 cells (2 × 10^6 cells/mouse) and reached their peak (an average of 250 colonies or more) at 12 days after inoculation. However, very few pulmonary lesions were observed in BF1 mice 2 wk after inoculation with the same number of B16F1 melanoma cells. However, very few pulmonary lesions were observed in BF1 mice 2 wk after inoculation with the same number of B16F1 melanoma cells.

FIGURE 1. Pulmonary metastasis in mice various days after inoculation of 2 × 10^6 B16F10 (BF10 mice) or B16F1 melanoma cells (BF1 mice) per mouse. The metastatic colonies were counted on days 3, 6, 9, 12, and 14 after the tumor inoculation in BF10 mice (●) or BF1 mice (○). Values are the average number of pulmonary colonies obtained from five mice. Results are the mean ± SE.

Next, the generation of Th2 cells in these two groups of mice was examined. The CD4+ T cells from BF1 mice and BF10 mice were assayed for their ability to produce IL-4 in vitro. When splenic CD4+ T cells from BF10 mice at 7 and 14 days after tumor inoculation were stimulated in vitro with anti-CD3 mAb (2.5 μg/ml), 1956 to 1517 pg/ml IL-4 were produced into the supernatants (Fig. 2). However, splenic CD4+ T cells from normal mice (naive CD4+ T cells) or CD4+ T cells from BF1 mice at 3, 7, and 14 days after the tumor inoculation did not produce IL-4 following Ab stimulation. The CD4+ T cells from the spleens of mice at 14 days after inoculation with B16F10 cells (2 × 10^6 cells/mouse) were designated as BF10-Th2 cells and used in the following experiments. As shown in Table I, following stimulation with anti-CD3 mAb, BF10-Th2 cells produced IL-4 and IL-10 in their supernatants. However, IL-2 and IFN-γ were not produced by these cells following stimulation with the mAb, whereas naive T cells stimulated with the mAb produced only type 1 cytokines.

Adoptive transfer of BF10-Th2 cells to BF1 mice

To examine the role of BF10-Th2 cells on pulmonary metastasis, adoptive transfer of CD4+ T cells from BF10 mice (donors) to BF1 mice (recipients) was performed, and the metastatic colonies in lungs of the recipient mice were counted, as described above.

FIGURE 2. Production of IL-4 by CD4+ T cells from BF1 mice or BF10 mice. For induction of IL-4, CD4+ T cells (2 × 10^6 cells/ml) prepared from spleens of BF1 mice or BF10 mice on days 3, 7, and 14 after tumor inoculation were stimulated in vitro with anti-CD3 mAb. As a control, CD4+ T cells from normal mice (naive CD4+ T cells) were stimulated with mAb. Culture supernatants, harvested 48 h after stimulation, were assayed for IL-4 by ELISA. Results are the mean ± SD of triplicate determinations.

Abbreviations used in this paper: BF1 mice, mice implanted with B16F1 melanoma cells; BF10 mice, mice implanted with B16F10 melanoma cells; BF10-Th2 cells, CD4+ T cells from BF10 mice 14 days after the tumor inoculation.
BF10-Th2 cells were prepared from spleens of mice at 14 days after inoculation of 2 \times 10^6 cells/mouse of B16F10 melanoma cells. When 5 \times 10^6 cells/mouse of BF10-Th2 cells were adoptively transferred to BF1 mice, the number of metastatic colonies was 229 \pm 45, as compared with 23 \pm 6 colonies in lungs of BF1 mice inoculated with naive CD4 \(^+\) T cells. The numbers of colonies in recipient mice increased in response to the numbers of BF10-Th2 cells transferred (Fig. 3). However, when variable numbers of CD4 \(^+\) T cells from BF1 mice or naive CD4 \(^+\) T cells were adoptively transferred to recipient mice, the pulmonary spread did not occur (Fig. 3). These results indicate that the BF10-Th2 cells generated in BF10 mice accelerated the development of pulmonary metastasis in BF1 mice.

**Phenotypic characterization of the BF10-Th2 cells**

CD4 \(^+\) T cells from BF10 mice at 14 days after the tumor inoculation (BF10-Th2 cells) were treated with various mAbs followed by complement. The remaining cells were assayed for their ability to produce IL-4. When BF10-Th2 cells were treated with anti-CD11b, anti-CD28, or anti-TCR-\(\alpha\)\(\beta\) mAbs followed by complement, the IL-4-producing cells were depleted (Table II). However, BF10-Th2 cells treated with anti-TCR-\(\gamma\)\(\delta\) mAb and complement produced 2380 pg/ml of IL-4 into their supernatant. These results indicate that BF10-Th2 cells were CD4\(^+\)CD11b\(^-\)CD28\(^-\)TCR-\(\alpha\)\(\beta\)\(^+\) Th2 cells.

**Role of IL-4 on pulmonary metastasis**

The IL-4-producing activity of BF10-Th2 cells was demonstrated by the above experiment. Therefore, in the next studies, the role of IL-4 secreted from BF10-Th2 cells on pulmonary metastasis of B16 melanoma in BF1 mice was examined. When BF1 mice were injected i.p. with 0.1 to 10 \(\mu\)g/kg IL-4, pulmonary metastasis was increased in relation to increasing IL-4 amounts administered (Fig. 4). When BF1 mice were treated with a 10-\(\mu\)g/kg dose of IL-4, pulmonary metastasis in BF1 mice was increased to that observed in mice inoculated with 5 \times 10^6 BF10-Th2 cells/mouse (see Figs. 1 and 4). In contrast, pulmonary metastasis in BF10 mice or in BF1 mice inoculated with BF10-Th2 cells was reduced to the same level observed in BF1 mice when they were treated with anti-IL-4 mAb (250 \(\mu\)g/kg) (Table III). These results suggest that the IL-4 released from BF10-Th2 cells was important in the degree of pulmonary metastasis in mice inoculated with B16 melanoma cells.

**Discussion**

Metastatic tumors disseminate to organs and/or tissues distant from a primary tumor through the circulatory or lymphatic systems. The only tumor cells that propagate successfully are those capable of overcoming complex structural and functional barriers present in the host (19). Metastasized tumor cells are often inaccessible or are more resistant to conventional treatments for malignancy such as surgical excision, chemotherapy, and radiation treatment. It has been suggested that the immune response in hosts...
bearing benign tumors and malignant tumors are expressed using distinct patterns of cytokine production (12). The cytokines produced in hosts bearing benign tumors have been shown to be type 1, whereas type 2 cytokines are produced in hosts bearing malignant tumors. Recently, several reports (27–31) have described a shift from type 1 T cell responses to type 2 T cell responses in individuals bearing progressive malignant tumors. Ghosh et al. (27) reported that the production of IL-4 increased preferentially in mice bearing experimental solid tumors. Ascitic fluids from mice implanted with EL-4 thymoma contained TGF-β and IL-10 (32), whereas type 2 cytokines are produced in hosts bearing malignant tumors. Recently, several reports (27–31) have described a more distinct pattern of cytokine production than that seen in studies where IL-4 was administered (27). The antitumor activity of IL-4 demonstrated using gene therapy was more distinct as compared with studies where IL-4 was administered directly (51–53).

When tumor growth is accelerated by IL-4 and type 2 T cell influences, T cells appear to be major effector cells (14–16). However, T cells may not be involved when tumor growth is inhibited by IL-4 (43–45), IL-4-transfected cells (46) or IL-4 gene transfer (51–53). The antitumor activity of IL-4-transfected cells and IL-4 gene therapy has been demonstrated in T cell-deficient nude mice (40, 44, 51). IL-4 has been shown to be an inducer of VCAM in the tumor microvasculature (54). This suggests that eosinophils with a ligand for VCAM may be allowed to bind effectively to tumor cells following exposure to IL-4. Tepper et al. (43) reported that eosinophils infiltrated into tumor sites were shown to be effector cells since the IL-4-transfected cells were rejected. In a subsequent report using athymic nude mice (44), they described prominent eosinophils that infiltrated into tumor sites and IL-4-mediated antitumor effects were linked. However, it is not clear what conditions are required to enhance tumor growth by IL-4 (or type 2 T cell responses) or IL-4-associated tumor rejections to be activated. The tumor-specific antimetastatic activity of lymph node cells from mice inoculated with IL-4-transfected B16 melanoma cells was enhanced when compared with those of effector cells from mice inoculated with parental B16 melanoma cells (47–49). Other activities also exist for IL-4. It up-regulates protein expression and enzymatic activity of aminopeptidases, which may relate to the tumor cell invasion process and metastasis (50), and stimulates systemic antitumor immunity in hosts bearing B16 melanoma cells (47–49). Furthermore, IL-4 gene therapies against malignancy are receiving increased attention (51–53). The antitumor activity of IL-4 demonstrated using gene therapy was more distinct as compared with studies where IL-4 was administered directly (51–53).

### Table III. Effect of anti-IL-4 mAb on pulmonary metastasis in BF10 mice or BF1 mice inoculated with BF10-Th2 cells

<table>
<thead>
<tr>
<th>Mice</th>
<th>No. of Pulmonary Metastases</th>
<th>Reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BF10 mice received&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (control)</td>
<td>244 ± 22</td>
<td>0</td>
</tr>
<tr>
<td>Rat Ig</td>
<td>226 ± 37</td>
<td>7</td>
</tr>
<tr>
<td>Anti-IL-4 mAb</td>
<td>37 ± 16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>85</td>
</tr>
<tr>
<td>BF1 mice received&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>19 ± 5</td>
<td></td>
</tr>
<tr>
<td>BF10-Th2 cells (control)</td>
<td>347 ± 58</td>
<td>0</td>
</tr>
<tr>
<td>BF10-Th2 cells and rat Ig</td>
<td>326 ± 69</td>
<td>6</td>
</tr>
<tr>
<td>BF10-Th2 cells and anti-IL-4 mAb</td>
<td>60 ± 33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>83</td>
</tr>
</tbody>
</table>

<sup>a</sup> An average number of metastatic colonies per lungs of mice (10 mice each). Results are mean ± SE.

<sup>b</sup> At 4 and 6 days after inoculation with B16F10 melanoma cells (2 × 10<sup>5</sup> cells/mouse), mice (BF10 mice) were treated i.p. with a 250-μg/kg dose of anti-IL-4 mAb. As controls, the same mice were treated with saline or rat Ig (250 μg/kg).

<sup>c</sup> Three days after the implantation of B16F10 melanoma cells (2 × 10<sup>5</sup> cells/mouse), mice (BF1 mice) were treated with saline or inoculated with 5 × 10<sup>6</sup> cells/mouse of BF10-Th2 cells. BF10-Th2 cells were CD4<sup>+</sup> T cells prepared from spleens of mice at 14 days after the inoculation of 2 × 10<sup>5</sup> cells/mouse of B16F10 melanoma cells. BF1 mice inoculated with BF10-Th2 cells were treated i.p. with a 250-μg/kg dose of anti-IL-4 mAb or rat Ig 4 and 6 days after the tumor implantation.

References


